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Title: Empagliflozin improves primary haemodynamic parameters and attenuates the development of atherosclerosis in high fat diet fed APOE knockout mice.

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Abstract: The effects of long-term treatment with empagliflozin on biochemical and immunohistochemical markers related to atherosclerosis and atherosclerosis development in the aorta of apolipoprotein E knockout [Apo-E (-/-)] mice were evaluated in this study. Empagliflozin-treated mice had lower total cholesterol ($P<0.05$), fasting glucose ($P<0.01$), heart rate ($P<0.01$) and diastolic blood pressure (DBP) ($P<0.05$) compared to controls. Histomorphometry revealed reduced atherosclerotic lesion progress approaching statistical significance ($P=0.06$) and approximately 50% wider lumen area for the Empagliflozin treated mice group. Although empagliflozin significantly reduced VCAM-1 and MCP-1 ($P<0.05$, $P<0.01$, respectively) and marginally induced TIMP-1 and TIMP-2 mRNA expression ($P<0.08$, $P=0.1$ respectively), immunohistochemistry revealed a marginal reduction in VCAM-1 and MMP-9 ($P=0.1$) without affecting the expression of TIMP-2 and MCP-1 in atherosclerotic lesions. Empagliflozin improves primary haemodynamic parameters and attenuates the progression of atherosclerosis by reducing hyperlipidemia and hyperglycemia, while direct actions in aorta vessel mediated via SGLT-1 are strongly hypothesized

Empagliflozin improves primary haemodynamic parameters and attenuates the development of atherosclerosis in high fat diet fed APOE knockout mice

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Abstract

The effects of long-term treatment with empagliflozin on biochemical and immunohistochemical markers related to atherosclerosis and atherosclerosis development in the aorta of apolipoprotein E knockout [Apo-E^(-/-)] mice were evaluated in this study. Empagliflozin-treated mice had lower total cholesterol ($P<0.05$), fasting glucose ($P<0.01$), heart rate ($P<0.01$) and diastolic blood pressure (DBP) ($P<0.05$) compared to controls. Histomorphometry revealed reduced atherosclerotic lesion progress approaching statistical significance ($P=0.06$) and approximately 50% wider lumen area for the Empagliflozin treated mice group. Although empagliflozin significantly reduced VCAM-1 and MCP-1 ($P<0.05$, $P<0.01$, respectively) and marginally induced TIMP-1 and TIMP-2 mRNA expression ($P<0.08$, $P=0.1$ respectively), immunohistochemistry revealed a marginal reduction in VCAM-1 and MMP-9 ($P=0.1$) without affecting the expression of TIMP-2 and MCP-1 in atherosclerotic lesions.

Empagliflozin improves primary haemodynamic parameters and attenuates the progression of atherosclerosis by reducing hyperlipidemia and hyperglycemia, while direct actions in aorta vessel mediated via SGLT-1 are strongly hypothesized.

Keywords: Empagliflozin, SGLT2i, Atherosclerosis, Inflammation, *APOE* knockout mice

Abbreviations

SGLT2: sodium glucose co-transporter2; SGLT2i: sodium glucose co-transporter2 inhibitor; T2DM: type 2 diabetes mellitus; *APOE*(-/-): apolipoprotein E knockout Apo-E(-/-); CV: cardiovascular; CVD: cardiovascular disease; MCP-1: monocyte chemoattractant protein 1; CD68: cluster of differentiation 68; MMP-2: matrix

metalloproteinase- 2; MMP-9: matrix metalloproteinase-9; TIMP-1: tissue inhibitor of metalloproteinases-1; TIMP-2: tissue inhibitor of metalloproteinases-2; ICAM-1: intercellular adhesion molecule 1; VCAM-1: vascular cell adhesion molecule 1; Empa: empagliflozin; ECM: extracellular matrix; HbA1c: hemoglobin A1c; Il-6: interleukin 6; HFD: high fat diet; DBP: diastolic blood pressure; SBP: systolic blood pressure.

1. Introduction

Type 2 diabetes (T2DM) prevalence rates have been increasing during the past decades, in parallel to the documented obesity epidemic [NCD-RisC, 2016; Zimmet *et al.* 2016]. T2DM comprises up to 90% of all diabetic cases in adults, with the most recent International Diabetes Federation (IDF) estimates indicating that 415 million adults (1 in 11 adults) have diabetes, a number predicted to reach 642 million (1 in 10 adults) by 2040 [IDF, 2015]. Furthermore, close pathogenic links exist between diabetes and cardiovascular disease (CVD), with CVD currently representing the main cause of morbidity/mortality in diabetic patients (up to 80% of all diabetic patients die from CVD-related events) [ESC 2013; Matheus *et al.* 2013]. It becomes evident that T2DM-related cardio-metabolic disease poses a significant challenge in clinical practice, requiring new effective treatment options which will lower the disease burden and particularly the associated CVD risk.

Despite advances in our understanding of T2DM pathophysiology, so far the applied glucose-lowering strategies have had little or no impact on CVD progression/outcomes in T2DM patients, whilst only a limited number of new medications have been added to the arsenal against the current diabetes epidemic [*e.g.* incretin mimetics and sodium/glucose cotransporter 2 (SGLT2) inhibitors] [Bolen S, *et al.* 2016; Thompson *et al.* 2016]. Moreover, the complete spectrum of effects of these new anti-diabetic agents has not been fully clarified yet. In this context, current research is focused on exploring the exact effects/outcomes of the new anti-diabetic medications [*e.g.* of glucagon-like peptide-1 (GLP-1) agonists and SGLT2 inhibitors] not only in controlling hyperglycaemia, but also on T2DM-related CVD. Indeed, following the lack of success

in significantly controlling CVD progression in T2DM with previous anti-diabetic medications and tight glycaemic control, there is now increasing evidence indicating that certain newer anti-diabetic agents, particularly GLP-1 agonists (e.g. liraglutide) and SGLT2 inhibitors (e.g. empagliflozin and dapagliflozin) will offer significant CVD benefits independent of glycaemic control [Thompson *et al.* 2016; Flory *et al.* 2016].

SGLT2 inhibitors constitute the newest class of anti-diabetic medications which act by increasing urinary glucose excretion, and, hence, improve glycaemic control independently of insulin secretion [Heerspink *et al.* 2016; Marx *et al.* 2016]. Recently, the EMPA-REG OUTCOME trial, a key CVD outcome trial, showed that empagliflozin significantly lowered the combined CVD endpoint of CVD death, non-fatal stroke and non-fatal myocardial infarction in T2DM patients with prevalent CVD [Zinman *et al.* 2015]. Moreover, empagliflozin unexpectedly induced a significant reduction in the individual endpoints of CVD death, heart failure hospitalization and overall mortality in this high CVD risk population of T2DM patients. Of note, a recent meta-analysis by Zelniker *et al.* showed that the benefits of SGLT-2 inhibitors are more pronounced in patients with established atherosclerotic CVD [Zelniker *et al.* 2019]. Thus, much research focus has been placed on elucidating the mechanisms responsible for these beneficial CVD effects of SGLT2 inhibition, which appear to extend beyond glucose control, potentially including mechanisms relating to weight loss, blood pressure lowering and sodium depletion, neuro-hormonal and renal haemodynamic effects, and effects on myocardial energetics/signalling [Heerspink *et al.* 2016; Marx *et al.* 2016].

In the present study, we aimed to investigate the long-term effect of empagliflozin using a dose of 10 mg/Kg/day on atherosclerosis development in the aorta of the *APOE*^(-/-) atherosclerosis mouse model focusing particularly on its role in local factors related to atheroma plaque stability. Furthermore, classic CVD risk factors such as hyperlipidemia, hypertension, weight gain and inflammation were evaluated.

2. Materials and Methods

137 2.1 Animals

138 *APOE*^(-/-) mice (C57BL/6J-ApoEtm1Unc) were originally purchased from “The Jackson
139 Laboratory” and bred in the animal facility of National and Kapodistrian University of
140 Athens. Mice were kept at specific pathogen free (SPF) controlled environment (22-
141 26 °C temperature, 40-60% humidity and 12h light/dark cycle). Animal experiments
142 were approved by the local Animal Care and Use Committee.

143 2.2 Experimental protocols

144 20 male *APOE*^(-/-) mice were kept on a standard rodent chow. At the age of 5 weeks,
145 mice were switched to high fat diet (HFD- mucedola-Italy) (20-23% by weight; 40-45%
146 kcal from fat) containing cholesterol (0.2% total). After 5 weeks on HFD, mice were
147 randomly divided into two groups (1) Empagliflozin-group 10 mg/kg/day (n=10)
148 administered orally by gavage, and (2) control-group (n=10) administered the same
149 volume of 0.5% hydroxypropyl methylcellulose /day (vehicle), via gavage. After
150 10 weeks of treatment with Empagliflozin or vehicle, mice were culled under
151 isoflurane anesthesia by transection of the diaphragm and, the aorta along with heart
152 were rapidly excised. Food intake and body weight were measured once weekly over a
153 period of 10 weeks. Blood glucose levels were measured after 8-10h fast via tail
154 puncture at baseline, before Empagliflozin/vehicle oral administration, once during
155 experiment (5 week) and before the end of the experiment. Empagliflozin was
156 purchased from MCE (Cat. No. HY-15409) and dissolved in 0.5% hydroxypropyl
157 methylcellulose (Sigma).

158 2.2.1 Blood pressure measurement

159 Blood pressure was measured using a computerized non-invasive tail-cuff system
160 (CODAs, Kent Scientific, USA) [Nasiri-Ansari *et al.* 2018] once at baseline, before
161 Empagliflozin administration began and once before culling animals as described
162 previously. All measurements are reported as mean values of heart rate, systolic and
163 diastolic blood pressure.

164 2.2.2 Serum analysis of biochemical parameters

165 Venipuncture was performed once before the onset of Empagliflozin administration
166 from the facial vein and once by heart puncturing after culling the mice. Serum
167 glucose, total-cholesterol, triglycerides, and HDL- and LDL-cholesterol levels were
168 determined using a dedicated autoanalyzer.

169 2.2.3 RNA isolation and real time PCR

170 Total RNA was extracted from fresh frozen aorta using NucleoSpin RNA Plus kit
171 (MACHEREY-NAGEL). Extracted mRNA was then reverse transcribed into cDNA using
172 the iScript cDNA synthesis kit (Bio-Rad). Real-time PCR analysis was performed as
173 described previously [Nasiri-Ansari *et al.* 2018]. The expression of Matrix
174 Metalloproteinase -2 and -9 (MMP-2 and MMP-9) and their inhibitors (TIMP-1 and TIMP-
175 2), IL-6, intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1
176 (VCAM-1) and monocyte chemotaxis protein 1 (MCP-1) was measured using
177 Luna® Universal qPCR Master Mix (New England Biolabs) on a CFX96 (Bio-RAD). The
178 sequences of primers used for RT-PCR analysis in this study are listed in Table 1. A
179 melting curve analysis was performed to confirm the specificity of qPCR products.
180 Fold-changes were calculated using the $2^{-\Delta\Delta Ct}$ method and were normalized against 18s
181 mRNA expression. All reactions were performed in triplicates and repeated at least
182 three times.

183 3. Histochemistry and immunohistochemistry

184 3.1 Quantification of atherosclerotic lesion area

185 Aortic tissues were fixed and embedded in paraffin. The 4-µm-thick sections were
186 stained with hematoxylin-eosin (H&E) and used for histopathological analysis whereas
187 Masson's trichrome stained sections were used to quantify tissue section's collagen
188 content. The degree of pathological changes was evaluated microscopically by
189 measuring the area of atheromatous plaque. Results are reported as the percentage
190 of the neointima area containing the lesion. Threshold was set and the positively

191 stained area for each histochemical stain was automatically calculated and then the
192 percent of the positively stained area to the total cross-sectional vessel wall area or
193 intimal plaque lesion area was reported. Plaque area analysis was carried out using
194 Image Pro Plus software version 5.1 (Media Cybernetics, Inc.).

195 3.1.1 Immunohistochemistry

196 All sections were deparaffinized at 60 °C. Antigen retrieval was performed using
197 citrate buffer (PH.6.0) for 7 min at 100 °C followed by blocking with normal goat
198 serum (CST, 5425S) for 1 h. Slides were then incubated with appropriate
199 concentration of primary antibodies against CD68 (ZYTOMED, MSK055), α -smooth
200 muscle actin (ZYTOMED, MSK030), MCP-1 (ACRIS, AM32136PU-N), MMP-2 (Proteintech
201 Group, 103732-AP), MMP-9 (Proteintech Group, 10375-2-AP) and their inhibitors TIMP-
202 1 (Santa Cruz Biotechnology, sc-21734) and TIMP-2 (Santa Cruz Biotechnology, sc-
203 21735), ICAM-1 (Santa Cruz Biotechnology, , sc-8439) , VCAM-1 (Santa Cruz
204 Biotechnology, sc-13160) followed by incubation with corresponding secondary
205 antibody conjugated to horseradish peroxidase (ZYTOMED, ZUC053-100) and visualized
206 by applying DAB (CST.8059P). All slides were counterstained with hematoxylin and
207 integral absorbance was examined under light microscope and results were quantified
208 using Image Pro Plus software version 5.1 (Media Cybernetics, Inc.). A positive tissue
209 control was used to ensure the specificity of antibodies used in this study.

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211 3.1.2 Statistical analysis

212 We used Student's t-test, Welch's test or Mann-Whitney test, after assessing the
213 normality of data distribution with the Shapiro-Wilk test and the equality of variances
214 with the Levene's test, for comparisons between EMPA and control animals regarding
215 quantitative variables, namely weight, heart rate, blood pressure, biochemical blood
216 test results, PCR results, and plaque area, lumen area and collagen content in
217 immunohistochemical analysis. We used the Chi-square test or the Fisher's exact test,
218 as appropriate, for comparisons between EMPA and control animals concerning

qualitative variables from immunohistochemical analysis, namely MMP-2, MMP-9, TIMP-1, α -ACTIN, CD-68, MCP-1, VCAM-1 and ICAM-1. We included a paired t-test for comparing the same parameter in each animal before and after intervention. All tests were two-tailed and results were considered statistically significant if P-value was less than 0.05. Statistical analysis was performed using the 23rd edition of Statistical Package for Social Sciences (SPSS) (IBM Corporation, Armonk, NY, USA).

4. Results

4.1 Oral administration of Empagliflozin for 10 weeks improved diastolic blood pressure, heart rate and reduced fasting blood glucose levels

No significant difference in daily food intake was observed between the two groups. Body weight was significantly increased in both groups after feeding HFD and 10 weeks of oral Empagliflozin/vehicle administration compared to the value measured at experiment baseline. No significant difference in weight gain was observed between Empagliflozin and control group (data not shown but available in supplementary material-supplementary Figure 1).

Fasting blood glucose (8 h of fasting) and serum lipid levels were measured before Empagliflozin/vehicle oral administration as well as at the end of intervention period. A significant reduction in glucose and total cholesterol levels ($P<0.01$ and $P<0.05$ respectively) along with a significant induction in HDL levels ($P<0.05$) were observed in Empagliflozin group at the end of intervention period compared to value measured at baseline. No significant changes in serum LDL was observed after 10 weeks of Empagliflozin/vehicle oral treatment (Figures 1.a & 1.b). Importantly, treatment with Empagliflozin, restored glucose levels to normal, contrary to placebo group where glucose increased significantly indicating progression to diabetes ($P<0.01$).

At the end of Empagliflozin/vehicle oral treatment, there was a significant difference from baseline only in fasting glucose ($P<0.01$) between the two groups. Mean \pm SD

changes in triglycerides, total cholesterol, LDL-, HDL-cholesterol and creatinine levels from baseline were not significantly different between the two groups (Figure 1.b)

Empagliflozin administration significantly reduced heart rate ($P \leq 0.01$), whereas no significant change was observed in the control group. In contrast, diastolic blood pressure values were significantly higher in the control group at experiment endpoint compared to values measured before the intervention ($P < 0.05$) (Figure 2.a). This finding was confirmed by comparing diastolic blood pressure and heart rate changes from baseline (values measured before onset of Empagliflozin/vehicle oral administration) between the two groups. ($P < 0.05$ and $P \leq 0.01$ respectively) (Figure 2.b). Ten weeks of Empagliflozin/vehicle intervention had no significant effect on systolic blood pressure (Figure 2.a).

4.2 Empagliflozin reduces atherosclerotic lesion area albeit has no effect on atherosclerotic plaque collagen content

Empagliflozin administration for 10 weeks significantly reduced atherosclerotic lesion progress. Six out of ten mice in the Empa-group and eight out of ten mice in the control-group developed atherosclerotic plaque.

Atherosclerotic plaque presence was assessed using H&E staining (representative Figure 3.a). Atherosclerotic lesion area was quantified measuring the percentage of lumen area covered by total plaque area in all aortic root sections, and the mean plaque area (\pm SD) was then calculated for each group (Figure 3.b). The lumen area was wider (by approximately 50%) in Empa-group compared with control-group ($P = 0.06$). Masson Trichrome staining showed that atherosclerotic lesions in both groups had similar collagen content ($P = 0.6$). Images and quantitative data are presented in Figures 3.a & 3.b.

4.3 Empagliflozin reduced the expression of inflammatory molecules and improved metalloproteinase profile

The effect of Empagliflozin treatment on the expression of inflammatory and adhesion molecules (IL-6, MCP-1 & ICAM-1, VCAM-1 respectively), matrix metalloproteinases (MMP-2, MMP-9) and their inhibitors (TIMP-1, TIMP-2), was investigated, isolating total RNAs from the aortic root and analyzed using real-time quantitative RT-PCR.

We demonstrate that oral Empagliflozin administration significantly reduces VCAM-1 and MCP-1 mRNA levels ($P<0.01$ and $P<0.05$ respectively) while it induces TIMP-1 mRNA expression levels trending towards statistical significance ($P=0.085$) (Figure 4.a). Empagliflozin treatment causes no significant alteration in IL-6, ICAM-1, TIMP-2, MMP-2 and MMP-9 mRNA levels compared to control group.

A balance between MMPs and TIMPs is known as an indicator of MMPs overall collagenolytic activity. To this end, TIMP-1/MMP-2 ratio mRNA levels were measured. Our findings demonstrate that TIMP-1/MMP-2 mRNA levels ratio was significantly higher in Empa-group ($P<0.05$) compared to control group. (Figure 4.b)

Immunohistochemistry staining of aortic root section revealed that Empagliflozin treatment marginally reduced CD-68 and MMP-9 protein levels while marginally induced TIMP-1 expression in atherosclerotic lesions compared to the control group ($P=0.1$) (Figure 5).

Although an increase in the expression of α -ACTIN, TIMP-2, and a borderline decrease in MCP-1, MMP-2, and ICAM-1 active-protein expression was observed in Empa-group compared to control group, the differences were not statistically significant (data not shown but available in supplementary material-supplementary Figure 2).

5. Discussion

Although clinical trials have showed the anti-atherogenic effects of SGLT2-i in patients with T2DM, providing data for primary and secondary prevention for CVD, the exact mode of their direct and/or indirect actions mediating this effect is not fully explored yet [Heerspink *et al.* 2016; Marx *et al.* 2016]. Interestingly, according to the

EMPA-REG OUTCOME Trial, reductions in key CV outcomes and mortality with empagliflozin vs placebo were consistent across the wide spectrum of CV risk [Fitchett *et al.* 2018].

Herein, we investigated the long term effect of empagliflozin using a higher dose of 10mg/Kg/day, contrary to what has been used by others previously [Han *et al.* 2016] on atherosclerosis development in the aorta of APOE^(-/-) mice with high fat diet-induced atheromatosis.

In contrast to human studies [Lee *et al.* 2018], empagliflozin did not reduce body weight in APOE^(-/-) compared to control group. Both groups, consuming similar amounts of food, gained weight without significant differences among them. Previous studies have showed that empagliflozin for 8 weeks decreased body weight in APOE^(-/-) mice at a dose 1-3mg/Kg/day, while it did not affect significantly the weight of ZDF rats (type 2 diabetes model) at a dose of 10 and 30mg/Kg/day [Han *et al.* 2016, Steven *et al.* 2017].

Interestingly, a recent study of an orally administered small molecule (LX4211) that inhibits both intestinal SGLT1 and renal SGLT2, and improves glycaemic control in both humans and mice, showed divergent effects regarding body weight, increasing it in mice - through hyperphagia- but not in humans [Powell *et al.* 2014]. Apart from species-specific differences and differences in animal models used (obese, non-obese, diabetic, atherosclerotic etc.) other mechanisms implicating dose- and time-dependent effects on energy expenditure and/or activity could also provide possible explanations for these conflicting results.

In our study, total cholesterol decreased, and HDL-cholesterol increased following long-term administration of empagliflozin. Regarding HDL-cholesterol, our data are in line with previous studies demonstrating that empagliflozin given at a lower dose of 3mg/Kg/day - but not 1mg/Kg- for 8 weeks in APOE^(-/-) mice, resulted in increased HDL. No changes were observed in LDL-cholesterol. Interestingly, most clinical studies using various SGLT-2i reported increased LDL levels, while animal studies resulted in conflicting results [Kusaka *et al.* 2013, Leng *et al.* 2016, Steven *et al.* 2017, Nakatsu *et al.* 2017, Filippas-Ntekouan *et al.* 2018, Ji *et al.* 2017].

333 According to a recent work by Basu *et al.* [2018] SGLT2 inhibition, leads to increased
334 LDL-cholesterol via reducing clearance of LDL from circulation.

335 Interestingly, triglyceride levels were not changed although the study by Han *et al.*,
336 showed that lower doses of empagliflozin for 8 weeks led to decreased serum
337 triglycerides. These contrasting results could be attributed to the higher doses and
338 the longer duration of treatment. We assume that higher doses of empagliflozin could
339 result in SGLT1 binding which is known to regulate glucagon secretion [Han *et al.*
340 2016]. It has been demonstrated that higher expression of SGLT1 in islets is associated
341 with higher glucagon secretion [Suga *et al.* 2019]. Although empagliflozin has the
342 higher selectivity for SGLT2 among other SGLT2i [Anker *et al.* 2018], in high serum
343 concentrations such those achieved by administration of 10mg/Kg/day, could
344 decrease glucagon secretion via inhibiting SGLT1 in α cells. A decrease in glucagon
345 levels, a hormone that suppresses *de novo* synthesis of triglycerides in the liver and
346 attenuates NAFLD [Wang *et al.* 2016], could counteract the favorable effects of
347 empagliflozin on insulin sensitivity [Karen *et al.* 2016]. Although, we didn't measure
348 glucagon levels, we found increased hepatic steatosis and inflammation in the Empa-
349 group compared to control-group, strengthening this notion (unpublished data).

350 Herein, we demonstrated a beneficial effect on DBP while SBP remained unchanged.
351 Although clinical trials have demonstrated the beneficial effects of empagliflozin on
352 blood pressure reducing daytime, 24-hour, morning home and clinic SBP at 12 weeks
353 [Kario *et al.* 2018], animal studies have yielded conflicting results [Terami *et al.*
354 2014, Terasaki *et al.* 2015, Ishibashi *et al.* 2016, Habibi *et al.* 2017, Hammoudi *et al.*
355 2017, Tahara *et al.* 2018]. Empagliflozin given at the same dose as in our study but
356 over a shorter duration (5 weeks), improved glycemic control but didn't decrease
357 either SBP or DBP [Aroor *et al.* 2018].

358 A possible explanation for our findings is the empagliflozin-induced increase in eNOS
359 activity [Aroor *et al.* 2018], an effect that could result in the decrease in DBP. It has
360 been previously reported that eNOS gene exerted effects on DBP [Zhu *et al.* 2005].
361 Moreover, empagliflozin has been reported to promote cardiac diastolic relaxation via
362 modulation SGK1/ENaC profibrosis signaling and associated interstitial fibrosis [Habibi

et al. 2017, Lin *et al.* 2014]. It is important to note that recent study by Xu *et al.* [Xu *et al.* 2019] showed that mice fed high fat diet for a period of 16 weeks increased their diastolic pressure while systolic pressure remained unchanged. Thus, it seems that empagliflozin managed to counteract the detrimental effects of high fat diet on diastolic blood pressure.

We demonstrated additionally, a significant decrease in heart rate with empagliflozin. A recent study in humans showed that a decrease in resting heart rate at the end of 24 weeks of treatment with tofogliflozin was not correlated with changes in body weight, HbA1c and SBP but with changes in insulin resistance [Matsubayashi *et al.* 2018]. Thus, although we did not measure insulin levels, it can be hypothesized, that the decrease in heart rate we demonstrate could be attributed to the suppression of sympathetic nervous system activity by amelioration of hyperinsulinemia. Indeed, empagliflozin has been found by others to reduce insulin levels and improve insulin resistance estimated by HOMA-IR model [Kusaka *et al.* 2016].

Although the number of mice with atherosclerotic lesions did not differ significantly between the two groups, there was a marginal reduction that almost approached significance in aortic root plaque area (approximately by 50%-P=0.06) in Empa-group compared to controls. The beneficial effects on lipids profile and DBP could contribute to the decrease in plaque formation. Attenuation of atherosclerosis process could also be attributed to the glucose-lowering effects of empagliflozin. Nevertheless, Han *et al.*, reported that the beneficial sequelae of empagliflozin on atheromatosis in APOE^(-/-) mice were exerted irrespective of achieved glucose homestasis, since the group treated with glimepiride had similar glycemic control without improvement in atheroma formation [Han *et al.* 2016]. The same study reported a beneficial effect of empagliflozin on atheroma formation in the aortic root (with 25% reduction) when given at a dose of 1 and 3mg/Kg/day for 8 weeks in APOE^(-/-) mice. Herein, we showed that treatment with a higher empagliflozin dose of 10mg/Kg/day over a longer duration (10 weeks), may further reduce atheroma burden (approximately by 50%); an important factor contributing to this additional effect of empagliflozin could be the time of treatment initiation in relation to atheroma

formation. In the experiment by Han *et al.*, mice were fed a HFD diet 13 weeks prior to the intervention with empagliflozin -vs 5 weeks in the present study-, and one could assume that the intervention at earlier stages of atheromatosis could contribute to more pronounced effects. Of note, we investigated the mRNA expression of SGLT-1 and SGLT-2 in aorta vessel and we found that SGLT-1 was expressed in all samples while SGLT-2 was faintly detected in two of the samples (data not shown). Thus, it could be strongly hypothesized that direct effects of empagliflozin are mediated via SGLT-1 inhibition.

MCP-1 is important mediator of the atherosclerosis process [Harrington *et al.* 2000]. A marked suppression of the local inflammatory cytokine expression may be the central mechanism involved. Of note, local gene silencing of MCP-1 expression, turned a vulnerable plaque into a more stable plaque phenotype in APOE^(-/-) mice [Liu *et al.* 2012]. In our study, expression of MCP-1 mRNA was reduced in Empa-group compared to controls, although immunohistochemistry revealed no significant changes. There are no studies investigating the effect of empagliflozin on MCP-1 expression locally in the atherosclerotic lesion, to date. Oezle *et al.* [2014] found lower MCP-1 expression in aortas excised from STZ-induced diabetic rats treated with 30mg/Kg/day, but not with 10mg/Kg/day empagliflozin for seven weeks compared to untreated; however, this model is not ideal for studying atherosclerosis.

Another study showed that empagliflozin has no effect on MCP-1 transcription in renal cortex compared to control group, irrespective of glucose levels [Gangadharan Komala *et al.* 2014], while Han *et al.* using APOE^(-/-) mice demonstrated decreased serum MCP-1 levels as well as decreased MCP-1 mRNA expression in adipocytes following empagliflozin administration for 8 weeks. Interestingly, *in vitro* experiments in human endothelial cells indicated that empagliflozin did not suppress IL-1 β -stimulated MCP-1 mRNA expression, whereas canagliflozin did so [Mancini *et al.* 2018]. In our previous work, canagliflozin given for 5 weeks reduced significantly the expression of MCP-1 protein in atheroma lesion of APOE^(-/-) mice [Nasiri-Ansari *et al.* 2018].

In addition, we found that long-term administration of empagliflozin reduced the expression of VCAM-1 mRNA, albeit, this effect was not confirmed at protein level, similar to MCP-1 [Nasiri-Ansari *et al.* 2018].

In vitro experiments have shown that empagliflozin had no effect on TNF α induced secretion of ICAM-1 and VCAM-1 in HUVEC cells [Uthman *et al.* 2018], while Oezle *et al.* found decreased ICAM-1 mRNA expression in non-atherosclerotic aorta from STZ-induced diabetic rats treated with 30mg/Kg/day.

Immunohistochemistry staining revealed that treatment with empagliflozin marginally reduced CD-68 and MMP-9 protein levels while marginally induced TIMP-1 expression in atherosclerotic lesions compared to control group. However, plaque collagen content did not differ significantly between the two groups. In line with was is reported by Han *et al.*, empagliflozin did not affect significantly plaque stability as evaluated by collagen content and plaque smooth muscle cells (SMCs). However, there was a trend for reduced MMP-9 protein and increased TIMP-1/MMP-2 protein ratio in Empa-group compared to controls. It is possible that factors other than the ones measured herein influence more potently plaque stability.

An important advantage of our study is that we evaluated factors (MMPs, TIMPs, MCP-1, ICAM-1, VCAM-1) known to play an important role in plaque vulnerability at both mRNA and active protein level in relation to the end point (i.e collagen content and SMCs), directly on atheroma plaque. Interestingly, we found equivocal results regarding MCP-1 and ICAM-1, highlighting the necessity of the evaluation of active protein apart from the transcripts. A possible explanation for this discrepancy could be an increased degradation rate of mRNA compared to protein [Panganiban *et al.* 2014]. Scarce literature is available on the impact of SGLT2-i on plaque stability, and in most available studies, these factors have been evaluated at mRNA level only and/or in artery sections not corresponding to plaque lesion, giving thus misleading results [Nakatsu *et al.* 2017; Leng *et al.* 2016; Han *et al.* 2016].

5.1 Conclusions

450 Although more mechanistic studies need to be performed, our study suggests that
451 empagliflozin has anti-atherogenic properties affecting traditional CVD factors such as
452 HDL-cholesterol, hypertension and heart rate, though not inducing weight loss.
453 Undoubtedly, glucose lowering effects contribute to this result; however, there is
454 sufficient evidence in the literature, suggesting that empagliflozin may have
455 pleiotropic actions. In this study, we commenced empagliflozin administration after 5
456 weeks of diet manipulation, when atherosclerosis process was established, and plaque
457 formation was initiated. This therapeutic design was chosen to enhance the validity
458 and fidelity of a model of secondary CVD prevention. We hypothesized that an earlier
459 intervention may be more potent in attenuating the process of atheromatosis.
460 Compositional changes in plaque like SMCs and collagen content were not found in the
461 empagliflozin group confirming the absence of significant alterations in
462 metalloproteinase system and inflammation. Moreover, studies with a longer
463 duration, and/or earlier initiation of treatment in relation to initiation of
464 atherosclerosis process (resembling a mouse-model for the study of primary
465 prevention of CVD) will shed more light in the therapeutic potential of empagliflozin.

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468 **Competing interests:**

469 The authors declare that they have no competing interests.

470 **Ethics approval and consent to participate:**

471 This study was approved by the Athens University Medical School Ethics Committee
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676 **Figure 1.**

677 Total serum cholesterol, serum HDL, serum LDL and fasting blood glucose levels in Empa-group
678 and Control-group before and after 10 weeks of Empagliflozin/vehicle oral administration.
679 1a. Empa oral administration led to a significant reduction in total cholesterol and fasting blood
680 glucose levels and a significant induction in HDL levels while had no effect on serum LDL levels.
681 Fasting glucose levels were significantly increased in the control group at the end of
682 intervention. 1b. Fasting blood glucose changes from baseline were significantly different
683 between Empa- and Control-groups. Data are shown as mean±SD (**P< 0.01, *P<0.05)

684 **Figure 2.**

685 The effect of empagliflozin oral administration on diastolic, systolic blood pressure and heart
686 rate of APOE(-/-) mice. 2a. 10 weeks of empagliflozin intervention significantly reduced heart
687 rate while had no effect on systolic and diastolic blood pressure. A significant increase in
688 diastolic blood pressure was observed in the control group after 10 weeks on intervention.
689 2b. Diastolic blood pressure and heart rate changes from baseline were significantly different
690 between two groups. Data are shown as mean±SD (**P<0.01, *P<0.05)

691 **Figure 3.**

692 Empagliflozin oral administration for 10 weeks marginally reduced atherosclerosis progress in
693 APOE(-/-) mice. Arrows indicate atherosclerotic lesion sites. 3a. Representative images of H&E,
694 Masson staining of aortic root. 3b. Quantification of free lumen area is shown as a percentage
695 of free lumen by the total area of aortic root. The plaque collagen content was measured via
696 quantification of Masson trichrome positive area over complete plaque area. Values are shown
697 as mean±SD. Original magnification ×40.

698 **Figure 4.**

699 VCAM-1, MCP-1 and TIMP-1 mRNA levels (n=5) in aortic root of APOE(-/-) mice treated with
700 empagliflozin/vehicle for 10 weeks. 4a. VCAM-1 and MCP-1 mRNA expression was significantly
701 reduced in Empa-group, while TIMP-1 mRNA expression was marginally induced. 4b. TIMP-
702 1/MMP-2 mRNA ratio was also significantly elevated in Empa-group (compared to Control-
703 group). Data are shown as mean±SD (**P< 0.01, *P<0.05)

704 **Figure 5.**

705 Immunohistochemical analysis of atherosclerotic lesions on APOE(-/-) mice after empagliflozin
706 oral administration. 5a. Representative images from the aortic root, immunostained for VCAM-
707 1, TIMP-1, MMP-9 and CD68. 5b. Quantification of positive cell proportion stained for VCAM-1,
708 TIMP-1, MMP-9 and CD68. Positive cell proportion stained with each antibody was scored as
709 described previously. A marginal increase in TIMP-1 protein expression along with marginal
710 reduction in MMP-9 and CD68 expression were observed in Empa-group as compared to the
711 Control-group. Data are shown as mean±SD(P<0.1)(Original magnification ×40)

712 Supplementary figure 1.

713 Changes in body weight in both groups in response to treatment/vehicle.

714

715 Supplementary figure 2.

716 Immunohistochemical quantification of the positive cell proportion stained for α -ACTIN, MCP-
717 1, MMP-2, TIMP-2 and ICAM-1.

718

Empagliflozin improves primary haemodynamic parameters and attenuates the development of atherosclerosis in high fat diet fed APOE knockout mice.

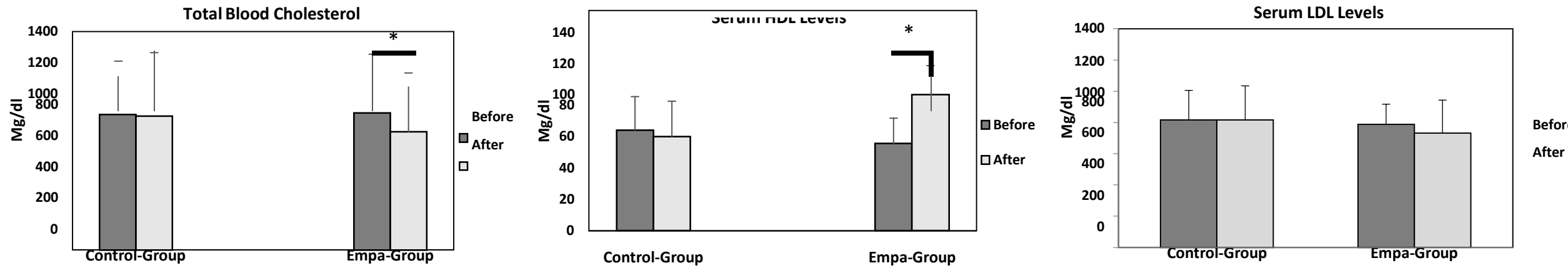
Highlights:

- Empagliflozin reduces heart rate and diastolic blood pressure in *APOE*^(-/-) mice.
- Empagliflozin reduces total cholesterol and increases HDL cholesterol.
- Empagliflozin reduces atherosclerotic lesion formation.
- Empagliflozin reduces VCAM-1 and MCP-1 inflammatory molecule expression.

Table 1.

<i>Target Gene</i>	<i>Primer Sequence (Forward)</i>	<i>Primer Sequence (Reverse)</i>
MMP-2	5'-CCCTCAAGAAGATGCAGAAGTTC-3'	5'-TCTTGGCTTCCGCATGGT-3'
MMP-9	5'-CGTCGTGATCCCCACTTACT-3'	5'-AACACACAGGGTTTGCCTTC-3'
TIMP-1	5'-GCATGGACATTTATTCTCCACTGT-3'	5'-TCTCTAGGAGCCCCGATCTG-3'
TIMP-2	5'-TTCCGGGAATGACATCTATGG-3'	5'-GGGCCGTGTAGATAAACTCGAT-3'
MCP-1	5'-GCATCCACGTGTTGGCTCA-3'	5'-CTCCAGCCTACTCATTGGGATCA-3'
IL-6	5'-CCTCTGGTCTTCTGGAGTACC-3'	5'-ACTCCTTCTGTGACTCCAGC-3'
ICAM-1	5'-GGTTCTCTGCTCCTCCACAT-3'	5'-CCTTCCAGGCTTTCTCTTTG-3'
VCAM-1	5'-CTTCCAGAACCCTTCTCAG-3'	5'-GGGACCATTCCAGTCACACTTC-3'
SGLT-1	5'-AAGATCCGGAAGAAGGCATC-3'	5'-CAATCAGCACGAGGATGAAC-3'
SGLT-2	5'-GCAACATCGGCAGCGGTCAT-3'	5'-GCGGAGGTACTGAGGCATTGTG-3'
18S rRNA	5'-GTTCCGACCATAAACGATGCC-3'	5'-TGGTGGTTGCCCTCCGTCAAT-3'

Figure 1.a



1.b

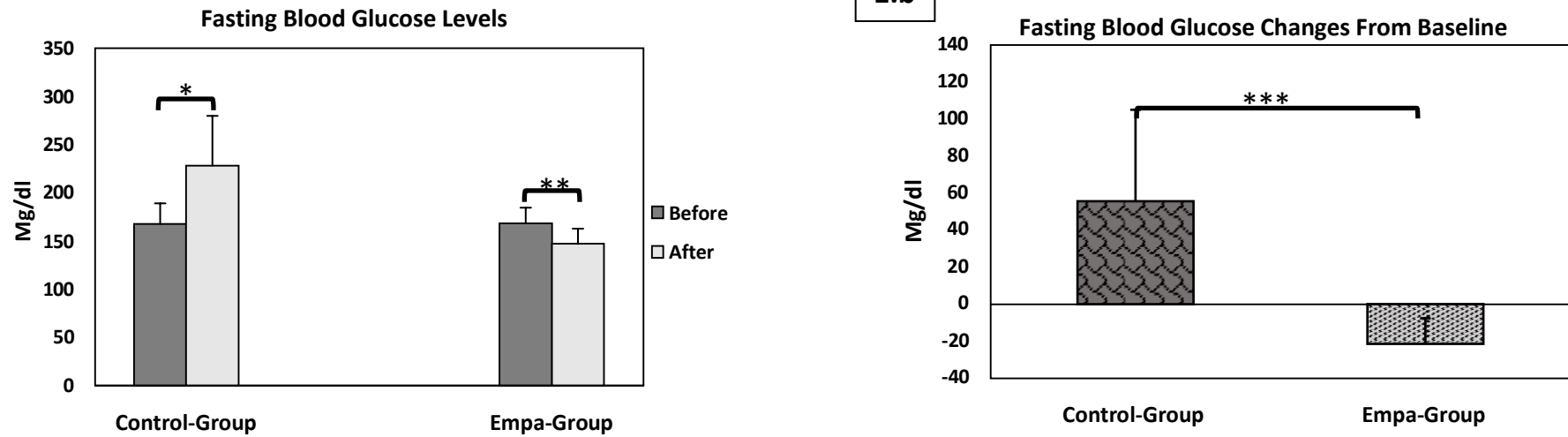
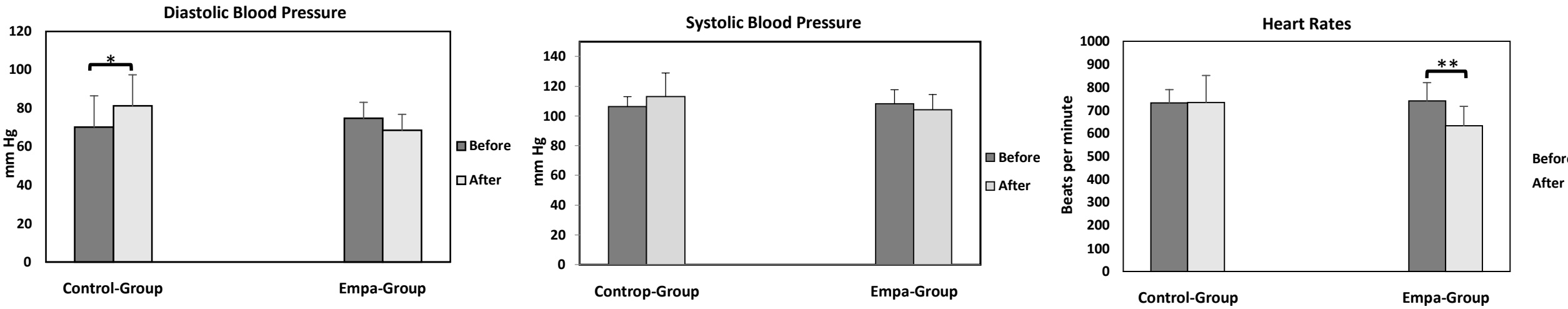
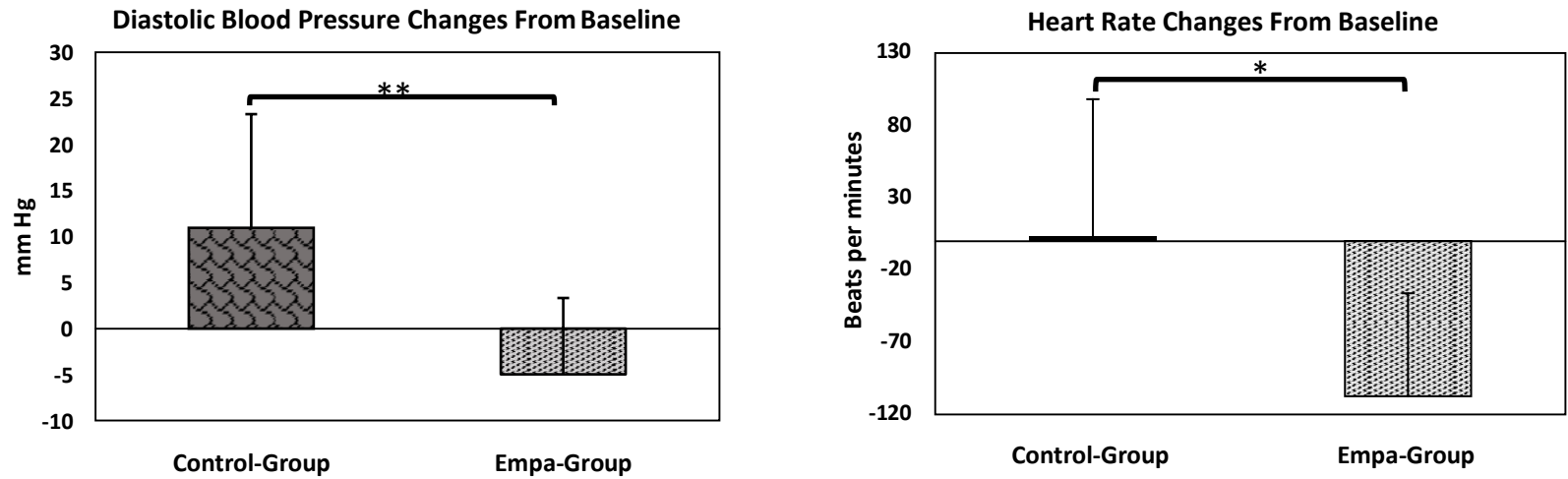


Figure 2.a



2.b



Figure

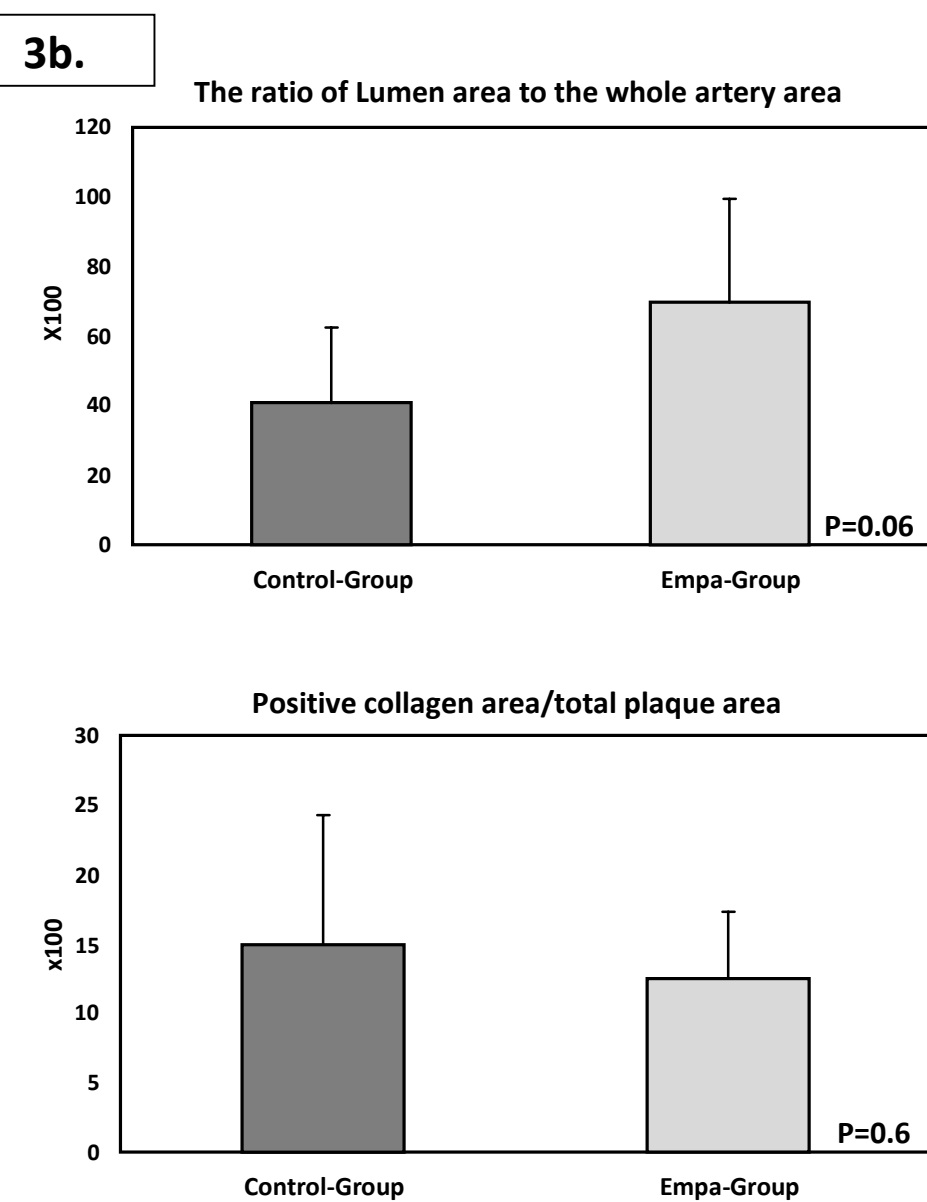
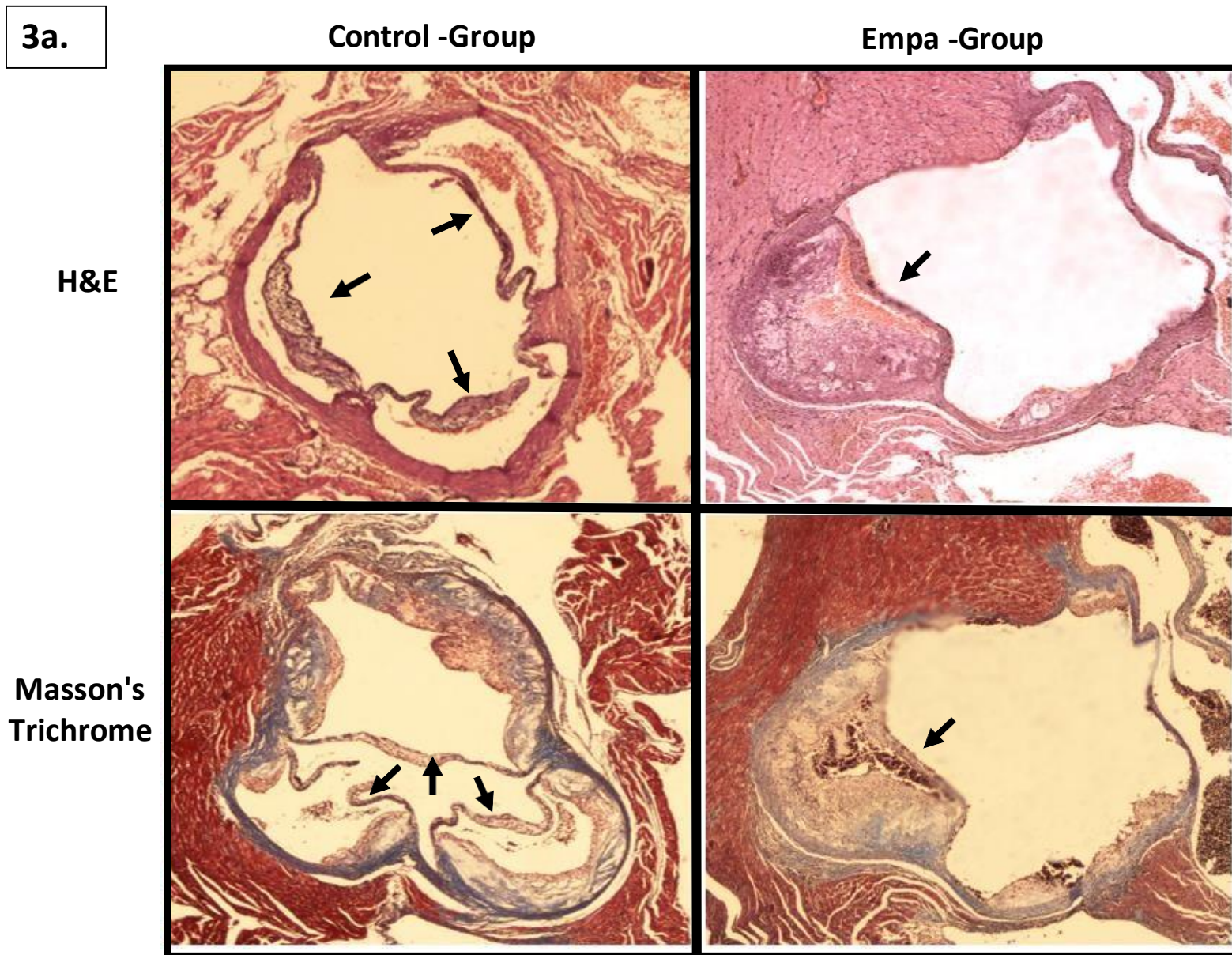
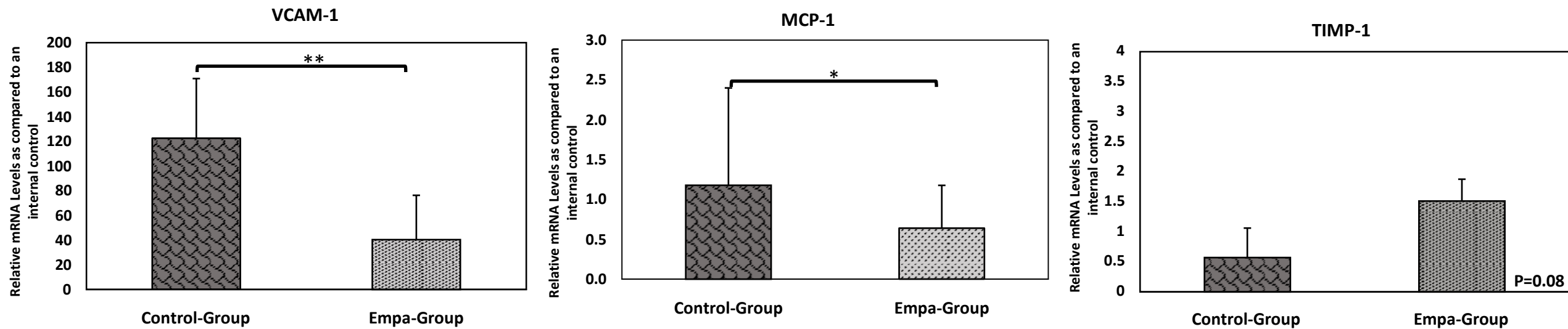


Figure 4.a



4.b

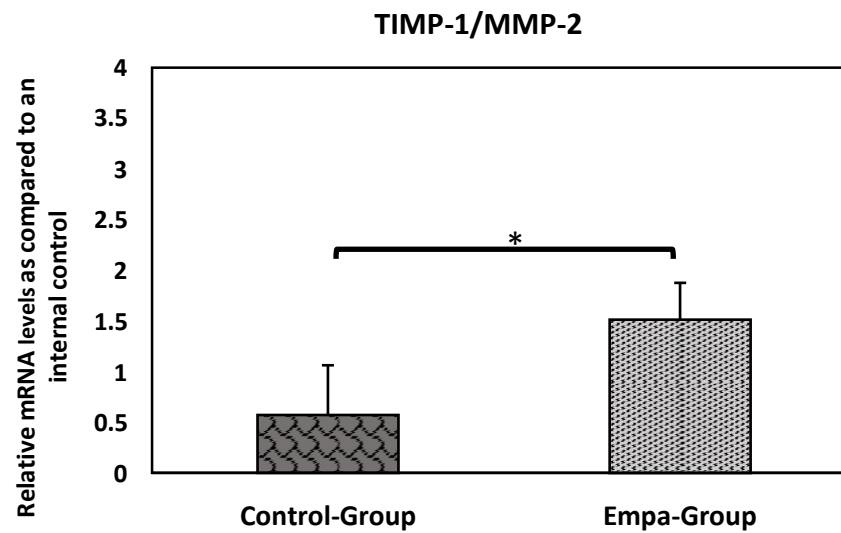


Figure 5.a

VCAM-1

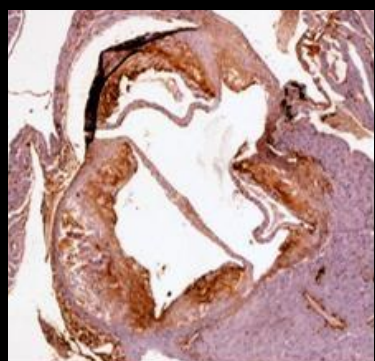
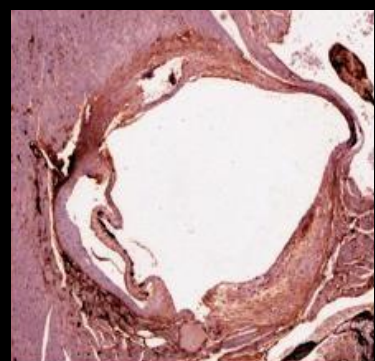
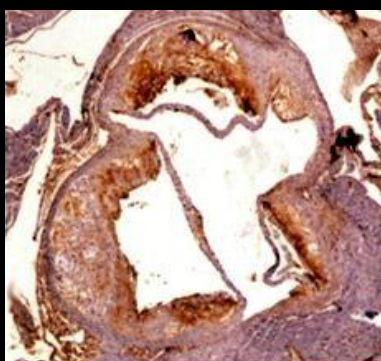
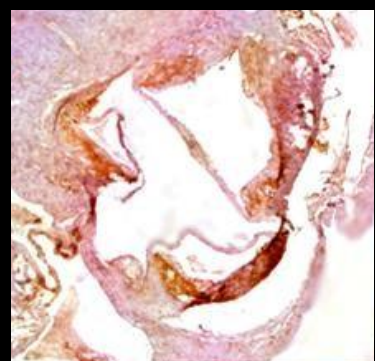
TIMP-1

MMP-9

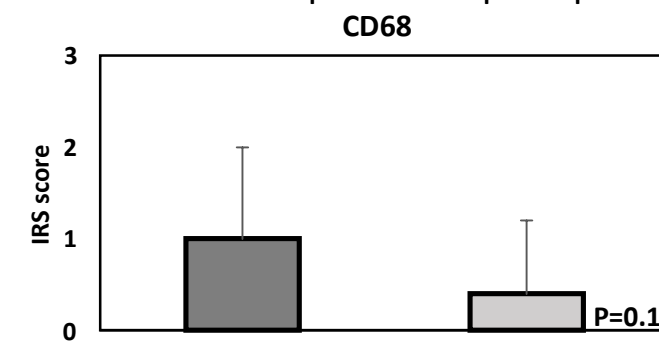
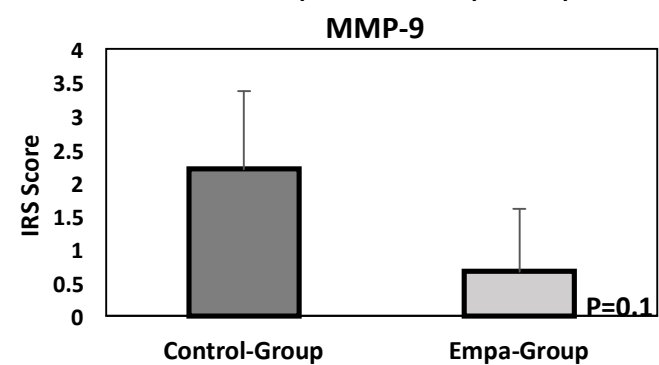
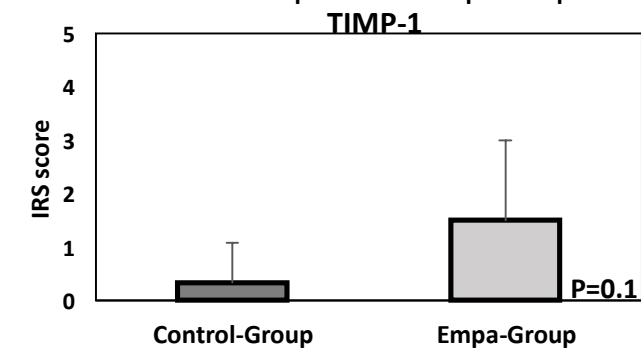
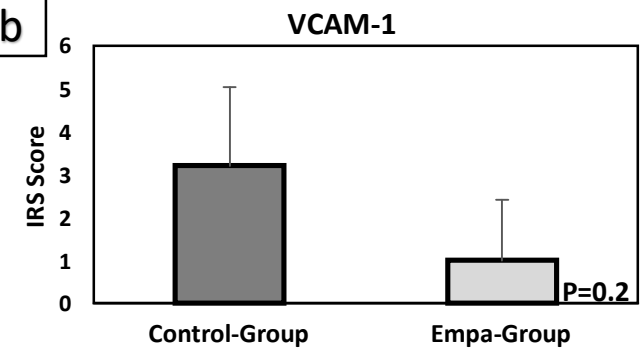
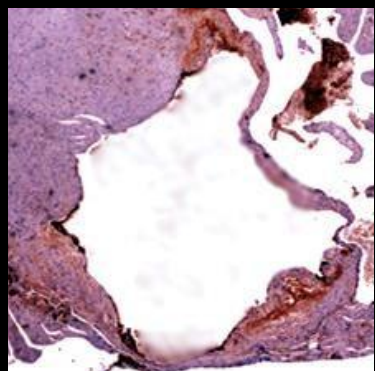
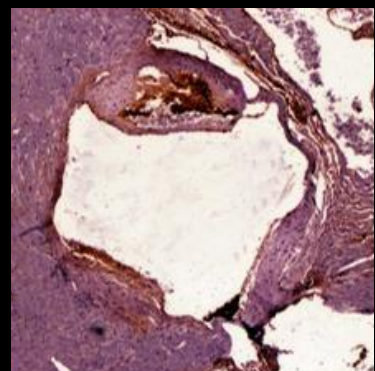
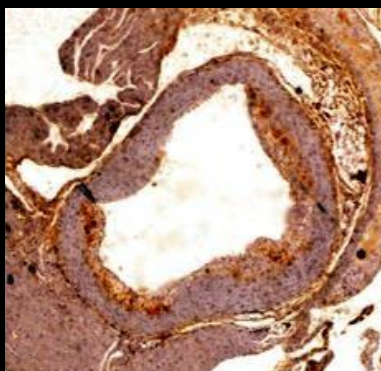
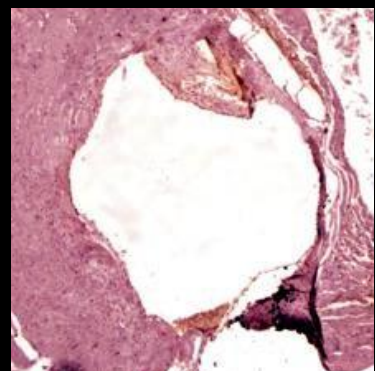
CD68

5.b

Control



Empa



Control-Group

Empa-Group

Human – Type 2
Diabetes



Treated with SGLT-2i
Anti-atherosclerotic
mechanism?

CVD Risk

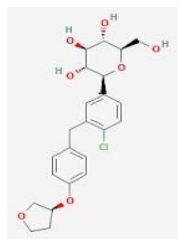
Mechanistic Murine Model



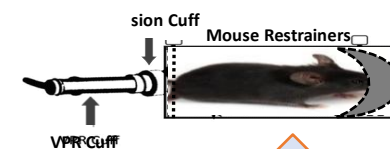
ApoE^{-/-} mice

5 weeks
HFD

Empagliflozin



Treated with 10mg/kg/day
Empagliflozin for
10 weeks



Heart Rate
Diastolic Blood Pressure

Progression of
atherosclerotic
lesion



Inflammatory
Markers

Cholesterol
Fasting Blood Glucose

HDL

Supplementary Material

[Click here to download Supplementary Material: supplementary-Fig-1.pdf](#)

Supplementary Material

[Click here to download Supplementary Material: supplementary.Fig.2 final.pdf](#)